PLATYPUS TECHNOLOGIES

Bringing Science to the Surface™

Oris™ Cell Migration Assay – TriCoated

Product No.: CMATR1 & CMATR5

96-well, 2-D Assay for Investigating
Cell Migration of Adherent Cell Lines
on Extracellular Matrix Components

PROTOCOL & INSTRUCTIONS

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Oris™ CELL MIGRATION ASSAY – TRICOATED

I. INTRODUCTION

The Oris™ Cell Migration Assay – TriCoated is a reproducible, sensitive, and flexible assay that can be used to monitor cell migration. Formatted for a 96-well plate, the assay utilizes Oris™ Cell Seeding Stoppers made from a medical-grade silicone to restrict cell seeding to the outer annular regions of the wells. Removal of the stoppers reveals a 2mm diameter unseeded region in the center of each well, i.e., the detection zone, into which the seeded cells may then migrate. The Oris™ Detection Mask is applied to the plate bottom and restricts visualization to the detection zones, allowing only cells that have migrated to be detected (see Figure 1). The Oris™ Cell Migration Assay – TriCoated is designed to be used with any commercially available stain or labeling technique. Readout can be performed by microscopy or use of a microplate reader.

The Oris™ Cell Migration Assay – TriCoated system has been designed for use with adherent cell cultures. This assay has been successfully used with HT-1080, PC-3, A549, NCI H1650, MDA-MB-231 and NMuMG cell lines.

Using the Oris™ Cell Migration Assay – TriCoated offers the following benefits:

- **Membrane-free Migration** - no transmembrane inserts to manipulate.
- **Reproducible Results** - the unique design provides well-to-well CV's < 12%.
- **Preserves Cell Morphology** - changes in cell structure can be monitored in real-time.
- **Versatile** - analyze data using multiple probes in a single well by using a microscope, digital imager, or fluorescence microplate reader.
- **Flexible** - perform kinetic or endpoint cell migration assays without the use of special instrumentation.
- **Optimize Migration** – measure cell migration directly on several different extracellular matrix coated surfaces all on one plate.

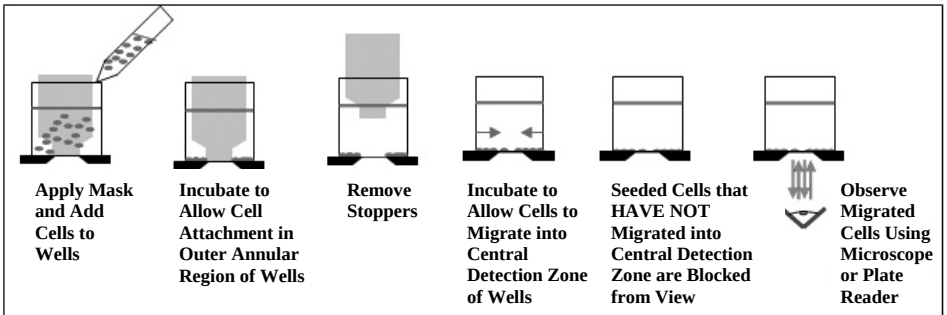


Figure 1. Schematic of Oris™ Cell Migration Assay – TriCoated

II. ORIS™ PLATE DIMENSIONS (per well)

Diameter of Well	6.5 mm
Diameter of Stopper Space (Detection Zone)	2 mm
Suggested Media Volume per Well (populated with Stoppers)	100 µl
Effective Area of Outer Annular Region (seeding region) per Well	30.03 mm^2
Effective Area of Central Detection Zone per Well	3.14 mm^2
Tri-Coated Plate Contents (see Figure 2):	
Columns 1-4	Uncoated, Tissue Culture Treated
Columns 5-8	Collagen I, Rat Tail
Columns 9-12	Fibronectin, Human
Storage Conditions	Refrigerate (4°C)

Important: Read Instructions Before Performing any Oris™ Assay.

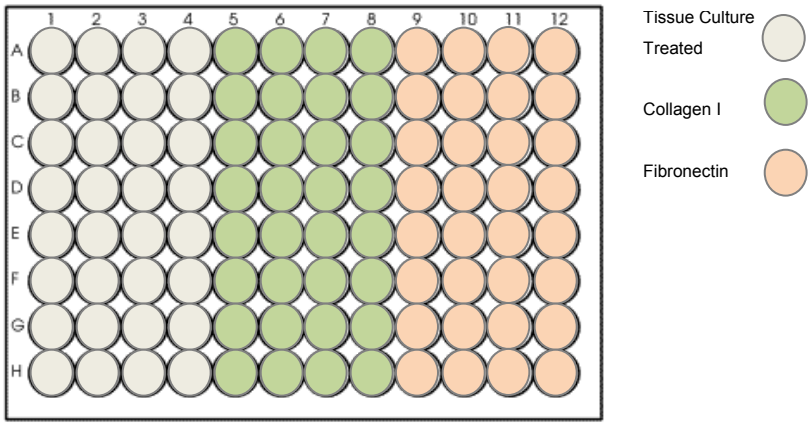


Figure 2. Layout of Coatings on Oris™ Cell Migration Assay – TriCoated plate

III. MATERIALS PROVIDED

Product No.: CMATR1

- Oris™ TriCoated, 96-well plate with Oris™ Cell Seeding Stoppers
  - 32 wells, Uncoated, Tissue Culture Treated
  - 32 wells, Collagen I coated
  - 32 wells, Fibronectin coated
- Oris™ Detection Mask, 1
- Oris™ Stopper Tool, 1

Product No.: CMATR5

- Oris™ TriCoated, 96-well plates with Oris™ Cell Seeding Stoppers, 5
  - 32 wells, Uncoated, Tissue Culture Treated
  - 32 wells, Collagen I coated
  - 32 wells, Fibronectin coated
- Oris™ Detection Mask, 1
- Oris™ Stopper Tool, 1

IV. MATERIALS REQUIRED

- Biological Cells
- Sterile PBS (containing both Calcium and Magnesium)
- Sterile FBS Containing Growth Medium
- Sterile Pipette Tips/Pipette or Multi-Channel Pipette
- Trypsin or Cell Scraper
- Inverted Microscope (optional)
- Fluorescence Microplate Reader (optional)
- Cell Culture Labeling Medium (phenol red-free/serum-free media)
- Cell Labeling Fluorescent Agent (eg., CellTracker™ Green, Calcein AM)
  - required if performing assay readout via microplate reader.

V. CELL MIGRATION ASSAY – TRICOATED PROTOCOL

The following steps should be performed in a biological hood using aseptic technique to prevent contamination.

1. Remove the Oris™ TriCoated Plate with Cell Seeding Stoppers from refrigeration and place on lab bench for ~1 hour to allow it to equilibrate to room temperature.

2. Visually inspect the underside of the populated 96-well plate to ensure that the Oris™ Cell Seeding Stoppers are firmly sealed against the bottom of the plate. To inspect the stoppers, turn the plate over and examine the stoppers for sealing (see Figure 3). If incomplete sealing is observed, return the plate to the upright position and use a sterile instrument to gently push the stopper back into the well until sealing is observed.

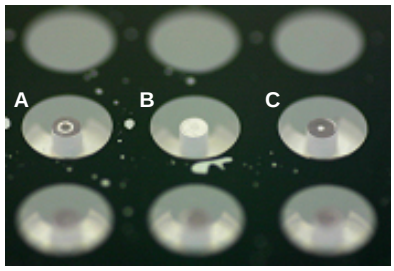


Figure 3. Stoppers that are:
A) Partially Sealed
B) Unsealed
C) Completely Sealed

NOTE: The sealing of the stoppers can be most easily observed if the plate is tipped at an angle and viewed under indirect light looking for the "bullseye" pattern at the bottom of each well.

3. Apply the Oris™ Detection Mask to the bottom of the 96-well plate if microplate reader data is being collected. The Detection Mask is not necessary if collecting imaging data.

   First Time Users: In order to prevent splashing of well contents, familiarize yourself with the attachment and removal of the Detection Mask before any liquids are placed into the wells:

   - Orient the chamfered corners of the mask with those of the 96-well plate, ensuring that the A1 corner of the mask is aligned with the A1 well of the plate (see Figure 4).
   - Align the holes in the attachment lugs with the bosses on the bottom of the 96-well plate.
   - Gently press the mask until it is flush with the bottom of the 96-well plate.

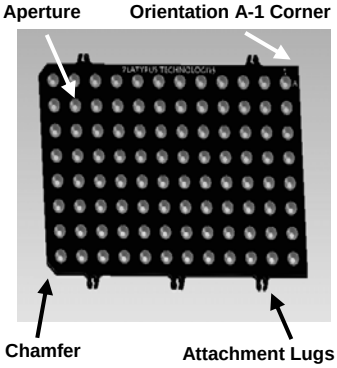


Figure 4. Features of Detection Mask

NOTE: It may be necessary to wash the mask with ethanol to remove dust and debris since the mask is not sterile. The mask may be applied at any point during the assay. For kinetic assays, it is often most convenient to apply the mask at the beginning of the assay before any liquids are placed in the well. For endpoint assays, using fixed and stained cells, it is often most convenient to apply the mask just before reading assay results.

4. If performing a kinetic analysis of cell migration, pre-stain cells with a fluorescent stain now.

5. Collect cells and prepare a suspension that is 10-fold greater in density than the optimal seeding concentration.

   First Time Users: The optimum seeding density of cells must be determined as an integral part of the design of the cell migration assay. Please refer to Appendix I for a discussion of this process.

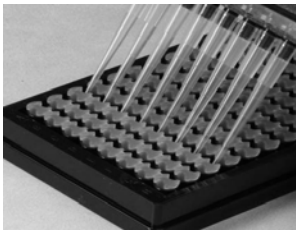


Figure 5. Media is Added with Single or Multi-Channel Pipette

6. Pipette 100 µl of suspended cells into each test well through one of the side ports of the Cell Seeding Stopper.

NOTE: For best results, add or extract media by placing the pipette tip along the wall of the well (see Figure 5). Care should be taken not to disturb the well coatings or the Cell Seeding Stoppers when introducing the pipette tip into the well. A slender/elongated tip or a gel loading tip may be useful.

7. **IMPORTANT:** Lightly tap the plate on your work surface to evenly distribute well contents (extreme tapping may result in splashing of well contents and lead to contamination).

8. Incubate the seeded plate containing the Oris™ Cell Seeding Stoppers in a humidified chamber (37°C, 5% CO_2) for 4 to 18 hours (cell line dependent) to permit cell attachment.

V. CELL MIGRATION ASSAY – TRICOATED PROTOCOL, continued

9. Remove plate from incubator.

10. Designate several 'reference' wells in which the stoppers will remain in place until results are read (t=0 pre-migration controls).

11. Using the Oris™ Stopper Tool, remove all other stoppers (see Figure 6).

NOTE: It may be necessary to wash the Oris™ Stopper Tool with 70% ethanol as the Stopper Tool is not sterile.

- Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the Oris™ Stopper Tool under the backbone of the stopper strip, keeping the underside of the Oris™ Stopper Tool flush with the top surface of the plate.
- Lift the Oris™ Stopper Tool ***vertically*** to gently remove the stoppers.

NOTE: DO NOT use the Oris™ Stopper Tool as a lever to pry the stoppers from the well (see Figure 6E), as doing so may cause displacement of seeded cells and may distort the detection zone area.

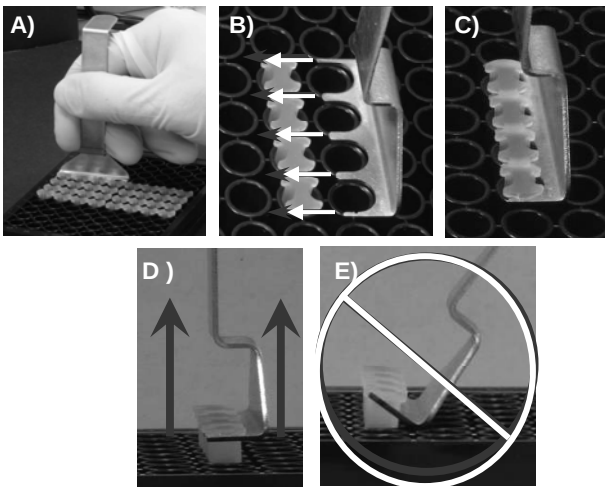


Figure 6 Removal of Stoppers. Panels A, B, and C) Position the Tines of the Stopper Tool between the Stopper Tips, D) Lift Vertically, and E) Do NOT Pry Stoppers

12. Remove media with a pipette and ***gently*** wash wells with 100 µl of sterile PBS (or media) to remove any unattached cells. Do not aspirate using an in-house vacuum.

13. Add 100 µl of fresh culture media to each well.

14. Incubate plate in a humidified chamber (37°C, 5% CO_2) to permit cell migration. Cells may be examined microscopically throughout the incubation period to monitor progression of migration. Migration time will vary depending upon cell type, experimental design, and ECM composition, as different ECM's have been shown to have varying effects on migration (even for a given cell line).

15. If performing an endpoint analysis of cell migration, stain cells with a fluorescent stain after sufficient migration has occurred. Refer to Section VI and Appendix II for further information on data acquisition and fluorescence staining technique.

NOTE: Oris™ Cell Seeding Stoppers are for single use only; Platypus can not guarantee the integrity of the stopper material after a second sterilization procedure.

VI. DATA ACQUISITION

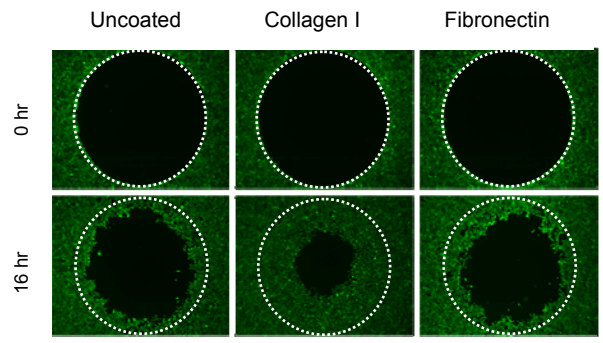
The readout of the Oris™ Cell Migration Assay – TriCoated can be conducted at any time, allowing the user to perform a kinetic assay or an endpoint assay. The Oris™ Cell Migration Assay – TriCoated is designed to be used with any commercially available stain or labeling technique. The readout can be performed by using a microscope, a microplate reader, or a High Content Screening or High Content Imaging Analysis platform.

Microscope Analysis

- Cell counting or image capture / analysis software, such as ImageJ freeware, available from NIH, can be used.
- Note: Microscopic observations are possible using phase contrast or bright field microscopy with colorimetric stains.
- No need to attach the Oris™ Detection Mask to the Oris™ microplate.

Microplate Reader Analysis

- Attach the Oris™ Detection Mask to the bottom of the Oris™ microplate (see Step 3 of Protocol).
- Optimal settings will vary according to the microplate reader make and model. Consult Appendix II and the equipment user manual for your particular instrument.
- The microplate reader MUST be set to read from the bottom of the plate.
- Sample data using a fluorescent stain and microplate reader analysis are shown in Figure 7. Wells with stoppers were seeded with 25,000 NMuMG cells/well (i.e., 100 µl at 2.5 x 10^5 cells/mL). The plate was incubated for 7 hours at 37°C, 5% CO_2. The stoppers were then removed from test wells. Stoppers were left in place in control wells until the staining step to serve as pre-migration controls. After an additional 16 hours to allow for migration, cells were fluorescently stained with Calcein AM for 30 minutes. Fluorescence was then measured using a microplate reader with the Detection Mask in place. The graph depicts the fluorescence signal present in the detection zones for each condition (mean ± S.D., *n* = 8 wells/condition). The images below, captured without a Detection Mask in place, illustrate representative data from pre-migration control wells and post-migration test wells.



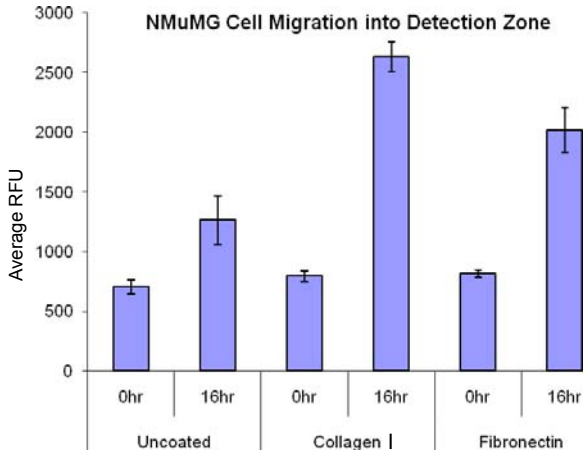


Figure 7. Cell migration data obtained using Calcein AM fluorescent stain.

VII. ORDERING INFORMATION

Product No.	Product Description	Package Size
CMATR1.101	Oris™ Cell Migration Assay - TriCoated, 1-pack: Oris™ TriCoated, 96-well plate with Oris™ Cell Seeding Stoppers, 1 32 wells, Uncoated, Tissue Culture Treated 32 wells, Collagen I coated 32 wells, Fibronectin coated Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	1-pack
CMATR5.101	Oris™ Cell Migration Assay - TriCoated, 5-pack: Oris™ TriCoated, 96-well plates with Oris™ Cell Seeding Stoppers, 5 32 wells, Uncoated, Tissue Culture Treated 32 wells, Collagen I coated 32 wells, Fibronectin coated Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	5-pack
CMA1.101	Oris™ Cell Migration Assay, 1-pack: Oris™ 96-well plate (black, clear bottom) with Oris™ Cell Seeding Stoppers, 1 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	1-pack
CMA5.101	Oris™ Cell Migration Assay, 5-pack: Oris™ 96-well plates (black, clear bottom) with Oris™ Cell Seeding Stoppers, 5 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	5-pack
CMACC1.101	Oris™ Cell Migration Assay – Collagen I Coated, 1-pack: Oris™ 96-well Collagen I coated plate (black, clear bottom) with Oris™ Cell Seeding Stoppers, 1 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	1-pack
CMACC5.101	Oris™ Cell Migration Assay – Collagen I Coated, 5-pack: Oris™ 96-well Collagen I coated plates (black, clear bottom) with Oris™ Cell Seeding Stoppers, 5 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	5-pack
CMAFN1.101	Oris™ Cell Migration Assay – Fibronectin Coated, 1-pack: Oris™ 96-well Fibronectin coated plate (black, clear bottom) with Oris™ Cell Seeding Stoppers, 1 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	1-pack
CMAFN5.101	Oris™ Cell Migration Assay – Fibronectin Coated, 5-pack: Oris™ 96-well Fibronectin coated plates (black, clear bottom) with Oris™ Cell Seeding Stoppers, 5 Oris™ Detection Mask, 1 & Oris™ Stopper Tool, 1	5-pack

To place an order, visit the Platypus Technologies website at: www.platypustech.com/order_main.html.
For technical assistance, contact Technical Support toll-free at (866) 296-4455 or techsupport@platypustech.com.

VIII. TERMS & CONDITIONS

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PLATYPUS shall not be liable for injury or damages resulting from the use or misuse of any of its products.

APPENDIX I: Determining Optimal Cell Seeding Concentration

This procedure is intended to assist in determining the cell seeding density needed to achieve confluency of your cell line when using the Oris™ Cell Migration Assay – TriCoated.

1. A suggested starting point is to evaluate three serial dilutions at the cell densities shown below. The cell seeding area of the well with the stopper in place is ~ 0.3 cm^2. Based on the typical seeding density of your particular cell line, you can infer a different cell number for your first serial dilution and adjust the numbers below accordingly.
2. Prepare a log-phase culture of the cell line to be tested. Collect cells and determine the total number of cells present.
3. Pellet cells by centrifugation. Prepare three serial dilutions at final concentrations of 1.0 x 10^6, 0.5 x 10^6 and 0.25 x 10^6 cells/mL.
4. Dispense 100 µl of suspension per well into the 96-well plate to result in the following plate layout:

Column	1	2	3
Cells / well	100,000	50,000	25,000
Number of wells	8	8	8

5. Incubate the plate in a humidified chamber (37°C, 5% CO_2) for 4 - 18 hours (cell line dependent) with cell seeding stoppers in place to allow the cells to firmly attach to the well surface.
6. Following cell attachment, remove the Oris™ Cell Seeding Stoppers from each well (see Figure 6) and ***gently*** wash the wells with PBS to remove non-attached cells.
   - Secure the 96-well plate by holding it firmly against the deck of your work space. Slide the tines of the Oris™ Stopper Tool under the backbone of the stopper strip, keeping the underside of the tool flush with the top surface of the plate.
   - Lift the Oris™ Stopper Tool ***vertically*** to gently remove the stopper. Do not use the Oris™ Stopper Tool as a lever to pry the stoppers from the well as doing so may cause displacement of the seeded cells.
7. Without a Detection Mask in place, use a microscope to visually inspect each well to determine the minimum cell seeding concentration that yielded a confluent monolayer at the perimeter of the detection zone.

At this point, if you plan to obtain the results of the Oris™ Cell Migration Assay – TriCoated via colorimetric or microscopic analysis, you have successfully determined the optimal cell seeding concentration to be used in Step 5 of the Cell Migration Assay – TriCoated Protocol.

APPENDIX II: Determining Optimal Fluorescence Plate Reader Settings

This procedure is intended to assist in optimizing your instrument settings when using a fluorescence microplate reader to capture data from the Oris™ Cell Migration Assay – TriCoated.

1. Using the optimal cell seeding concentration determined in Appendix I, perform a cell migration assay per Section V, Cell Migration Assay – TriCoated Protocol using culture conditions expected to result in robust cell migration. Be sure to include equal numbers of pre-migration control wells (stoppers left in place until staining) and post-migration test wells (stoppers removed after cell attachment period). A minimum of 8 wells per condition are recommended.
2. Perform the desired fluorescent staining technique.

   The Oris™ Cell Migration Assay – TriCoated has been designed to work with all types of fluorescent stains and staining techniques. The precise method for staining cells with fluorescent stains varies according to the nature of the individual stain. It is important to stain cells using a fluorescent reagent that uniformly stains cells. Probes affected by experimental conditions will increase variability of results and reduce correlation between fluorescence signal and cell migration. Please consult the manufacturer of your fluorescent stain for specific considerations.

   The following is an example Fluorescent Staining Protocol for using Calcein AM:

   a. To stain one fully-seeded 96-well plate, combine 5 µl of Calcein AM (1 mg/mL in dry DMSO) with 10 mL of phenol red-free and serum-free media or 1x PBS. Protect diluted Calcein AM solution from light until ready to use in step d.
   b. Carefully remove culture medium from wells.
   c. Wash wells with 100 µl of PBS (containing both Calcium and Magnesium).
   d. Add 100 µl of diluted Calcein AM solution to each well.
   e. Incubate plate at 37°C for 30 - 60 minutes.
   f. Attach mask and read promptly with microplate reader using appropriate filter set and sensitivity/gain settings (for a BioTek Synergy™ HT microplate reader, use 485/528 nm, sensitivity 55 nm).

3. If not already in place, apply the Oris™ Detection Mask to the plate. Using the bottom probe of a fluorescence microplate reader, obtain the fluorescence reading from each well. To achieve the optimal dynamic range, adjust the instrument settings (e.g., gain) to result in the greatest difference in fluorescence signal between pre-migration and post-migration wells. Refer to the instrument manual for your microplate reader for further guidance on instrument settings.

You have now successfully determined the optimal cell seeding concentration (to be used in Step 5 of the Cell Migration Assay – TriCoated Protocol) and microplate reader settings for analysis of cell migration using a fluorescence microplate reader.